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Inhibition of two enzyme systems in *Euchlanis dilatata* (Rotifera: Monogononta) as biomarker of effect of metals and pesticides

Juan Carlos Arias-Almeida[1], and Roberto Rico-Martínez[2]

[1]*Universidad Nacional Autónoma de México, Posgrado en Ciencias del Mar y Limnología, Ciudad Universitaria, Mexico, and* [2]*Universidad Autónoma de Aguascalientes, Centro de Ciencias Básicas, Departamento de Química, Aguascalientes, Mexico*

Abstract

The inhibitory effects on esterases and phospholipase A2 (PLA2) in the freshwater rotifer *Euchlanis dilatata*, native to Mexico, were assessed by fluorimetry after *in vivo* exposure (30 min) in laboratory conditions to sublethal concentrations of metals and pesticides. EC_{50} values for esterases ranged from 7.9×10^{-7} for DDT to 61.9 µg l^{-1} for methyl parathion, while corresponding values for PLA2 ranged from 0.96×10^{-6} for mercury to 69.2 µg l^{-1} for lead. These enzyme systems in *E. dilatata* are very sensitive to the tested agents and suggest they would be suitable biomarkers. However, sensitivity to other environmental contaminants should be investigated in laboratory conditions and field studies to assess their potential as environmental biomarkers.

Keywords: *Aquatic toxicology; esterases; phospholipase A2;* in vivo *biomarker; rotifers*

Introduction

The development of suitable methods for assessing contamination in aquatic environments has favoured biomarkers as a cost-effective strategy for detecting contaminant exposure and diagnosing sublethal effects (Silva & Pathiratne 2008). Biomarkers have been defined as quantitative measures of sublethal biochemical, behavioural, genetic or physiological changes resulting from individual exposure to xenobiotics (Choi 2004). A good biomarker needs high sensitivity, ease of measurement, to be dose- or time-dependent to toxicants, to have its variability understood and to be acceptable, and to be ecologically relevant (Mayer et al. 1992, Handy et al. 2003). Some enzyme systems commonly used as biomarkers of effect are esterases, β-galactosidases, glucosidases, phospholipase A2 (PLA2), lactate dehydrogenase and glutathione *S*-transferase (Burbank & Snell 1994, Castro et al. 2004). The sensitivity of esterases as target sites of inhibition of organophosphates, carbamates, detergents, organochlorines and metals is known (Rank et al. 2007). In addition, the extent of their inhibition in aquatic species is used as a biomarker of the neurotoxic effects of pollutants (Forget et al. 2003). PLA2s are ester hydrolases that cleave the sn-2 acyl ester bond from phosphatidylcholine, producing free fatty acids and lyso-glycerophospholipids. Arachidonic acid released from phospholipids in the cell and nuclear membrane is used as a substrate for biosynthesis of eicosanoids. These acids act as mediators in cell physiology and communication and coordinate the response to internal or external stimuli (Kitsiouli et al. 1999, García-Morán et al. 2008).

Metals such as Cd, Hg and Pb are common components of industrial and mining discharges in Mexico (Cervantes & Moreno-Sánchez 1999); they are toxic to aquatic organisms (Chapman et al. 2003) due to their high toxicity, persistence and ability to accumulate in the biota (Azevedo et al. 2009). Intensive agricultural use and campaigns to combat malaria have increased the input of pesticides into aquatic ecosystems (Castro-Castro et al.

Address for Correspondence: Juan Carlos Arias-Almeida, Universidad Nacional Autónoma de México, Posgrado en Ciencias del Mar y Limnología, Circuito Exterior s/n, Ciudad Universitaria, México D.F., C.P. 04510. Tel. (mob.) (52) 449 101 63 69. Fax: (52) 55 622 59 90. E-mail: jcariasa@hotmail.com

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2005). Mexico has not escaped to this tendency and both methyl parathion (Jaramillo-Juárez et al. 2009) and DDT (Mejía-Saavedra et al. 2005) are commonly used. Methyl parathion is extremely dangerous for its ability to cross biological membranes and be transformed into paraoxon (Pérez-Legaspi 2008). On the other hand, DDT is persistent in sediments (Walker et al. 2006) and has low selectivity (Valverde-Villarreal et al. 2001) which favours its bioaccumulation and toxic effects on the central nervous system of organisms (Devine & Furlong 2007).

Rotifers are recognized biological models in aquatic toxicology because of their small size, cosmopolitan distribution, high rates of population growth, ease of culturing in the laboratory and high susceptibility to environmental changes (Arora & Mehra 2003). In addition, rotifers possess a variety of enzymes with highly specific activities, which readily take up soluble substrates from their medium (Burbank & Snell 1994). Previous studies have shown that enzyme activity is a relevant ecotoxicological endpoint in toxicological tests with invertebrates (Choi 2004). Sublethal exposure to metals and organic compounds in copepods (Forget et al. 2003), *Daphnia magna* (Barata et al. 2004, 2007), *Brachionus calyciflorus* (Burbank & Snell 1994) and *B. plicatilis* (de Araujo et al. 2000) occurred an inhibition between 40 and 80% of glucosidase, esterase and PLA2 activities. Duquesne (2006) found that exposure of *D. magna* to sublethal concentrations of methyl parathion (<2.2 μg l^{-1}) caused esterase inhibition as well as individual and population effects. Pérez-Legaspi et al. (2002) and Pérez-Legaspi and Rico-Martínez (2003) found a high sensitivity (up to 400 000-fold for $HgCl_2$) of esterases and PLA2s to ten toxicants among three species of *Lecane*, suggesting that a species can be highly sensitive for a group of compounds, but insensitive to others. In this case, esterases were more sensitive than PLA2s for metals (Cd, Cu, Hg), in contrast to results found by Burbank and Snell (1994) for *B. calyciflorus*. Fluorescent methods used with invertebrates are based on the enzyme inhibition effect due to a particular toxicant, which is recorded as a reduction in fluorescence intensity in a dose-dependent manner (Garzón-López 2003). Therefore, the goal of this study was to assess the potential use of esterase and PLA2 activity as biomarkers of effect for exposure to metals and pesticides in *Euchlanis dilatata*, a periphytic species associated with aquatic plants and sediments of littoral areas (Segers 2008), where contaminants are generally deposited and become bioavailable.

Material and methods

The freshwater monogonont rotifer *E. dilatata* (lorica length: 193 ± 9.7 μm; lorica width: 140.8 ± 10.2 μm; mean ± SD, $n = 20$), was collected in a reservoir located at 21°44′13.7″N, 102°21′3.3″W and an altitude of 1838 m above sea level, in the city of Aguascalientes, Mexico. *E. dilatata* is a cosmopolitan species, periphytic, large and slow moving (Segers 2008). Cultures were maintained in EPA medium (96 mg $NaHCO_3$, 60 mg $CaSO_4.2H_20$, 60 mg $MgSO_4$ and 4 mg KCl l^{-1} deionized water), which is synthetic moderately hard water (80–100 mg $CaCO_3$ l^{-1}, pH 7.5), at a temperature of 25 ± 2°C and a photoperiod of 16:8 h of light:darkness in a bioclimatic chamber (Revco Scientific, Asheville, NC, USA). *E. dilatata* was fed every 2 days (1 ml) with the green alga *Nannochloris oculata* (strain LB2194, University of Texas Collection) grown in Bold´s Basal Medium (Nichols 1973) with a concentration of 1×10^6 cells ml^{-1}. Deionized water was obtained from a Water Pro System 18 MΩ (Labconco Co., Kansas City, USA). Hatching percentage of amictic eggs after 24 h under the culture conditions described above was >45%. The tested toxicants were reference chemicals of the highest purity available: (1) atomic absorption standards of cadmium and lead dissolved in 1% nitric acid [$Cd(NO_3)_2$ and $Pb(NO_3)_2$] from Sigma Co. (St Louis, MO, USA); (2) DDT and mercury chloride [$HgCl_2$] from Sigma Co.; (3) methyl parathion dissolved in acetone from Supelco Co. (Bellefonte, PA, USA). Nominal exposure concentrations (Cd: 0.1, 0.5, 1.0, 2.5, 5.0 and 10 μg l^{-1}; DDT: 10^{-9} to 10^{-5} μg l^{-1}; Pb: 1.0, 2.5, 5.0, 10.0, 15.0 and 25.0 μg l^{-1}; Hg: 1.0, 6.0, 12.0, 25.0, 50.0 and 100 μg l^{-1}; methyl parathion: 4.7, 9.4, 18.7, 37.5, 75.0 and 150 μg l^{-1}) were estimated between 0.3- and 0.5-fold the LC_{50} value previously determined in 48-h acute toxicity tests for each toxicant with neonates 24 h old. Values of 0.1-fold LC_{50}, did not establish significant differences between the treatments and the negative control (J.C.A.-A., 2010, unpublished data). Stock solutions (0.5, 10, 100 and 300 μg l^{-1}) were prepared in EPA medium (DDT and methyl parathion were previously dissolved in acetone), stored at 4°C in darkness and renewed each week. Fluorogenic substrates were prepared as follows. The substrate for esterases (cFDAam, 5-carboxifluorescein diacetate acetoxymethilester from Molecular Probes (Eugene, OR, USA) was prepared by diluting 1 mg of cFDAam in 1 ml of dimethyl sulfoxide (DMSO) making a stock that was divided in 50-μl aliquots and stored at -4°C. The substrate for PLA2 (PLA2, 2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine from Molecular Probes) was prepared by dissolving 5 mg of PLA2 in 0.5 ml of DMSO, making a stock divided into 50-μl aliquots and stored at -4°C.

The *in vivo* esterase inhibition tests were performed according to the protocol of Pérez-Legaspi et al. (2002) and the PLA2 tests to that of Pérez-Legaspi and Rico-Martínez (2003), both with slight modifications. Briefly, parthenogenetic eggs of *E. dilatata* were collected 24 h before the start of the tests and placed in 2-ml wells with EPA medium at pH 7.5 (US EPA 2002). Each test was

begun by the addition of 750 µl of EPA medium (negative control) or each toxicant concentration diluted in EPA medium into each well of a 24-well polystyrene plate (Costar Co., Cambridge, MA, USA). Then 25 neonates less than 24 h old were counted and transferred into each well using an Edmonson's plastic micropipette and an Olympus SZ61 stereoscope. Rotifers were exposed to a negative control and different toxicant concentrations in the dark at 25°C for 30 min in a bioclimatic chamber (Revco Scientific, Asheville, NC, USA). Previous exploratory analysis regarding the optimal exposure time for these tests has shown that a period of 30 min is satisfactory to achieve results quickly and that longer exposure (1 h) did not significantly increase the sensitivity of the test species (Burbank & Snell 1994, McDaniel & Snell 1999, Pérez-Legaspi et al. 2002). After the exposure period, 2 µl (5 µM) of fluorogenic substrate cFDAam was added to each well to assess the activity of esterases *in vivo*. For PLA2, 1 µl (17.2 µM) of PLA2 substrate was added to each well. The plate was gently shaken and then incubated in the dark at 25°C for 15 min. After the incubation with the substrate, 25 µl of 10% formalin solution were added to stop the enzyme activity and to fix the organisms. Although death occurs in neonates, the fluorescence intensity obtained is not significantly reduced a day after the test (Pérez-Legaspi et al. 2002, Pérez-Legaspi & Rico-Martínez 2003). Fifteen rotifers were randomly selected and transferred to a microscope slide containing two strips of tape positioned to support a cover-slide and to avoid damage to the neonates. Rotifers were observed at 10x magnification in a Leica DMLS inverted microscope. With an Infinity 3 camera and Infinity Capture 4.6.0 software (Lumenera Co., Ottawa, ON, Canada) photographic records were made of the fluorescence in each neonate, detecting higher intensity in the corona (for esterases) and mastax (for PLA2) of test organisms. The fluorescence was measured with an excitation spectrum from 450 to 490 nm and an emission barrier of 515 nm. The fluorescence intensity was measured by drawing a circle around the corona for esterases and around the mastax for PLA2, and obtaining the mean fluorescence value for these areas. A background value was also taken from the surroundings of the rotifer and subtracted from the previous fluorescence value. These operations were performed using image analysis with the software Kodak Digital Science 1D (Scientific Imaging Systems, Nashua, NH, USA). In total, 45 test organisms (15 per well in three replicates on different dates, $n=3$) were used for each control and toxicant concentration. The statistical analysis was performed with Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). We used one-way ANOVA and post-hoc comparison tests (Duncan) to determine the no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) points. Linear regression was used for calculating the EC_{50} (effect concentration where a 50% reduction in enzyme activity is observed) values, with the mean fluorescence and exposure concentrations as dependent and independent variables, respectively.

Results

The results of *in vivo* esterases and PLA2 inhibition tests obtained for *E. dilatata* exposed for 30 min to five toxicants are shown in Tables 1 and 2. The photographic records show the activity of esterases and PLA2 located in the corona and the mastax of organisms, respectively. There was a significance decrease in fluorescence intensity with increasing concentration of toxicants. EC_{50} values ranged from 7.9×10^{-7} to 61.9 µg l^{-1} for esterases, and from 0.96×10^{-6} to 69.2 µg l^{-1} for PLA2. Coefficients of determination, r^2 ranged from 0.75 to 0.96 for esterases and from 0.72 to 0.91 for PLA2. Data show a greater inhibition of both enzyme systems when there is exposure to the pesticide DDT in relation to metals and methyl parathion, observing the following order of sensitivity: DDT > Cd > Pb > $HgCl_2$ > methyl parathion. LOEC values showed that the response to adverse effects on enzyme activity appeared at low concentrations, indicating the high sensitivity of these enzyme systems in *E. dilatata* to the toxicants evaluated. The EC_{50}/LOEC ratio of cadmium was 11-fold. By contrast, the 50% enzyme inhibition value of methyl parathion occurred at a concentration 3.3-fold greater than LOEC. It indicates that the pesticide acts at a later point than the metal on test organisms, but with an immediate inhibitory effect. Likewise, lead, mercury and DDT have an EC_{50}/LOEC ratio of 4.2, 4.8 and

Table 1. Results of *in vivo* esterases inhibition tests in neonates of *Euchlanis dilatata* exposed to metals and pesticides during 30 min ($n=3$ replicates).

Toxicant	NOEC	LOEC	EC_{50}	CL95%	CV (%)
Cadmium	0.1	0.5	5.5	3.8–7.2	26.7
DDT	1×10^{-8}	1×10^{-7}	7.9×10^{-7}	6.21×10^{-7}–1.01×10^{-5}	21.7
Lead	2.5	5.0	20.8	17.5–24.0	2.0
Mercuric chloride	6.0	12.0	57.6	40.3–74.8	13.9
Methyl parathion	9.4	18.7	61.9	30.5–93.3	40.8

NOEC, no-observed-effect concentration; LOEC, lowest-observed-effect concentration; EC_{50}, effect concentration where a 50% esterase activity reduction is observed; CL95%, 95% confidence limits for EC_{50} value; CV, coefficient of variation. All values are in µg l^{-1}.

Table 2. Results of *in vivo* phospholipases A2 inhibition tests in neonates of *Euchlanis dilatata* exposed to metals and pesticides during 30 min ($n = 3$ replicates).

Toxicant	NOEC	LOEC	EC_{50}	CL95%	CV (%)
Cadmium	0.5	1.0	7.6	5.4–9.7	9.4
DDT	1×10^{-7}	1×10^{-6}	0.96×10^{-6}	4.63×10^{-8}–1.98×10^{-5}	30.4
Lead	1.0	2.5	14.1	8.7–18.5	41.9
Mercuric chloride	0.1	1.0	44.9	18.9–70.9	3.2
Methyl parathion	9.4	18.7	69.2	41.9–96.5	29.1

NOEC, no-observed-effect concentration; LOEC, lowest-observed-effect concentration; EC_{50}, effect concentration where a 50% phospholipase A2 activity reduction is observed; CL95%, 95% confidence limits for EC_{50} value; CV, coefficient of variation. All values are in $\mu g\ l^{-1}$.

7.9, respectively, possibly indicating a more effective mechanism of action compared with cadmium, and a rapid inhibitory effect on esterases once it reaches LOEC threshold. Figures 1 and 2 show the dynamics of inhibition of esterases and PLA2 for each toxicant evaluated. For DDT, we conducted a log transformation ($\log_{10}$) to low concentrations of exposure (10^{-9} to 10^{-5} $\mu g\ l^{-1}$) to obtain a better representation of the inhibitory effect.

The inhibitory action on PLA2 occurs at low concentrations, especially for DDT, whose real effect on 50% of individuals is achieved at a concentration 0.96-fold LOEC value. It suggests that sublethal effects are minimal or do not exist. Therefore, this enzyme system is not a good biomarker for this toxicant. Mortality of neonates is the most significant effect of exposure to DDT. For methyl parathion, lead and cadmium, EC_{50}/LOEC ratio is 3.7, 5.7 and 7.6, which suggests the occurrence of enzyme inactivation effect at a later point and close to the EC_{50} value. This result indicates that PLA2 would have little value as a biomarker of the effects of these toxicants. In contrast, the ratio for mercury (44.9) indicates an inhibitory effect from lower concentrations, even below the thresholds found for lead and methyl parathion. This suggests that PLA2 would be a good biomarker of effect in *E. dilatata* for exposure to mercury. Although EC_{50} values of both enzyme systems appear similar, it is possible to suggest that esterases are biomarkers more sensitive than PLA2 for cadmium and DDT, while PLA2 shows a greater sensitivity to the inhibitory effects of mercury. Likewise, the response of both enzyme systems suggests them as good biomarkers of effect with respect to methyl parathion.

Discussion

In vivo enzyme inhibition tests showed a high sensitivity of *E. dilatata* strain for exposure to the metals and pesticides evaluated, which was observed from lethal tests. EC_{50} values found for esterases and PLA2 (Tables 1 and 2) for toxicants are close to lethal concentrations. Therefore, these enzyme systems could not be considered good biomarkers of sublethal toxicity in our test organism. For example, *E. dilatata* presents a LC_{50}/EC_{50} ratio of 2.8 (Table 3) for $HgCl_2$, indicating that esterases would be 50% inhibited at a concentration 2.8-fold lower than required concentration for causing a 50% mortality of the test organisms. This result contrasts with the ratio of 400 000-fold found for three species of *Lecane* for the same toxicant (Pérez-Legaspi et al. 2002). Our test species results were more sensitive than *L. luna*, *L. quadridentata* and *L. hamata* for the metals evaluated, except for mercury where *L. luna* is 57 600-fold more sensitive than *E. dilatata*. The EC_{50} value of eight freshwater rotifers species (including *Asplanchna girodi*, *Keratella cochlearis*, *Plationus patulus*) reported by McDaniel and Snell (1999), divided by the EC_{50} value of *E. dilatata* (EC_{50}/EC_{50Ed} ratio) of esterases exposed to cadmium, suggests a greater sensitivity of our test species in a range between 7.3 (*Lepadella patella*) and 98.8 (*L. quadridentata*). Only *Trichocerca pusilla* (0.14) was more sensitive than *E. dilatata*. For pesticides, Pérez-Legaspi (2008) reports an EC_{50} value of 9400 $\mu g\ l^{-1}$ in *L. quadridentata* for esterase inhibition with methyl parathion, obtaining a LC_{50}/EC_{50} ratio (1.01) less than that of *E. dilatata* (Table 3). *B. calyciflorus* and *D. magna* showed an EC_{50} value of 5.57 mg l^{-1} and 0.075 $\mu g\ l^{-1}$, respectively, for their food filtration rate (*Nannochloris oculata*) after 5 h of exposure to methyl parathion (Fernández-Casalderrey et al. 1993). This would indicate a better sensitivity and rapid detection (30 min) of biochemical changes in *E. dilatata*, being more sensitive than rotifers and more tolerant than the cladoceran. With respect to PLA2, *E. dilatata* (Table 2) is sensitivity to lead and mercury, very similar to *L. luna* (LOEC: 0.01–75 $\mu g\ l^{-1}$; EC_{50}: 53–191 $\mu g\ l^{-1}$, Pérez-Legaspi & Rico-Martínez 2003). Our NOEC values show a wide range of sensitivity (10–2000-fold) for Cd, Pb and Hg in *E. dilatata* with respect to *B. calyciflorus* (Burbank & Snell 1994) and *Lecane* species (Pérez-Legaspi & Rico-Martínez 2003). According to these authors, esterases are more sensitive than PLA2s for cadmium and mercury. However, this trend could apply partially to *E. dilatata*, as PLA2s are more sensitive than esterases for mercury and lead. PLA2 inhibition in *L. quadridentata* for DDT indicates an EC_{50} value of 38.5 $\mu g\ l^{-1}$ (Mejía-Saavedra et al. 2005), which is 40 000-fold lower than the estimated sensitivity in *E. dilatata* (Tables 2 and 3).

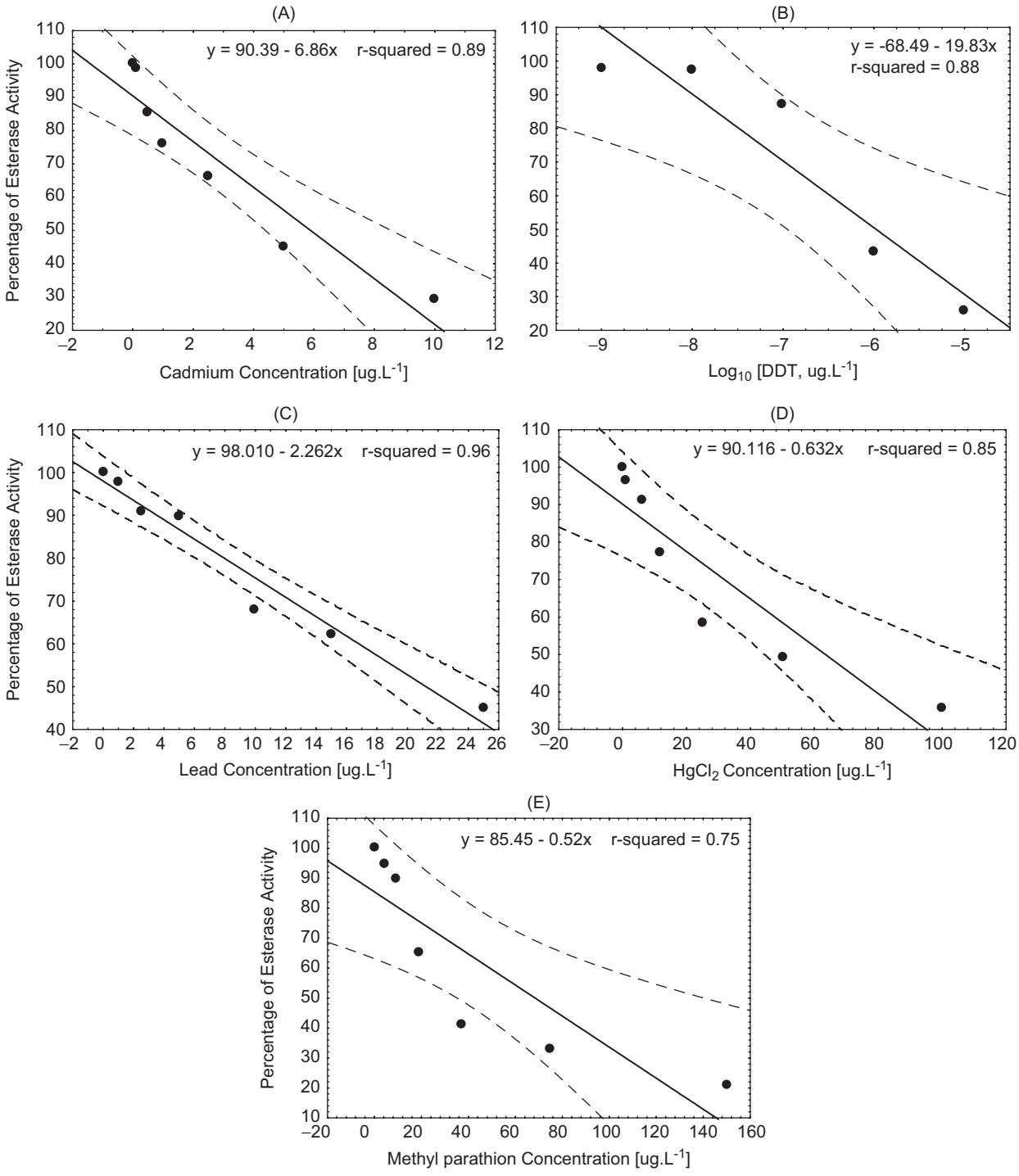

Figure 1. Dynamics of concentration-response curves for the esterase inhibition test with the rotifer *Euchlanis dilatata*. The black circles correspond to the mean values of 45 organisms exposed to nominal concentrations of (A) cadmium, (B) DDT, (C) lead, (D) mercury chloride and (E) methyl parathion. r-squared = coefficient of determination.

Despite the differential response of esterases and PLA2, sublethal response in both enzyme systems showed the same order of sensitivity to different toxicants: DDT > Cd > Pb > $HgCl_2$ > methyl parathion. The same dynamic was observed in the lethal tests (J.C.A.-A., 2010, unpublished data), indicating a homogeneous response in the test strain with respect to metals and pesticides. We found a lower sublethal effect of methyl parathion when compared with DDT in our study, in contrast to that reported in the literature regarding a greater acute toxicity of methyl parathion (Walker et al. 2006), despite its low persistence and bioaccumulation rate in the

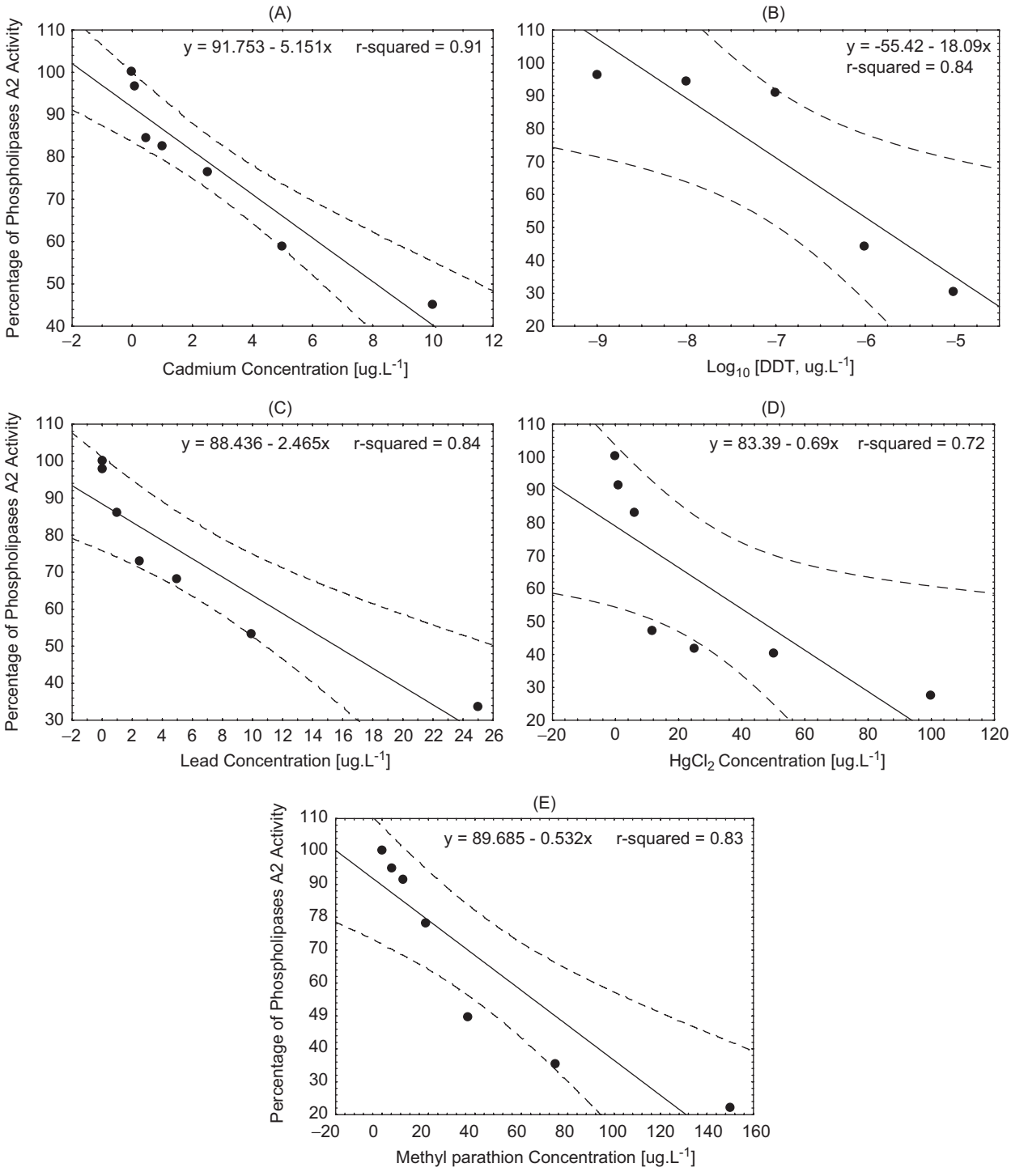

Figure 2. Dynamics of concentration-response curves for phospholipase A2 inhibition test with the rotifer *Euchlanis dilatata*. The black circles correspond to the mean values of 45 organisms exposed to nominal concentrations of (A) cadmium, (B) DDT, (C) lead, (D) mercury chloride and (E) methyl parathion. r-squared = coefficient of determination.

environment (Barata et al. 2007). Thus, exposure of our *E. dilatata* strain to environmental concentrations of DDT (992.6–49984 μg kg^{-1}) reported in sediments for diverse rivers in the southern Huastec region (Mejía-Saavedra et al. 2005) or concentrations of lead (90–130 mg kg^{-1}), mercury (3.8–4.8 mg kg^{-1}) and cadmium (1.23.0 mg kg^{-1}) in sediments of the San Pedro River in Aguascalientes, Mexico (Mora 2007), would probably result in the death of organisms, without allowing the establishment of populations of this species.

Table 3. Comparison between 48-h LC_{50} (lethal concentration) and 30-min EC_{50} values obtained by esterase-inhibition tests (EC_{50EST}) and phospholipases A2-inhibition tests (EC_{50PLA2}) for pesticides and metals in neonates of *Euchlanis dilatata*.

Toxicant	LC_{50}	EC_{50EST}	EC_{50PLA2}	LC_{50}/EC_{50EST}	LC_{50}/EC_{50PLA2}
Cadmium	18	5.5	7.6	3.3	2.4
DDT	2.67×10^{-4}	7.9×10^{-7}	0.96×10^{-6}	337.9	278.1
Lead	39	20.8	14.1	1.9	2.8
Mercuric chloride	164	57.6	44.9	2.8	3.6
Methyl parathion	607	61.9	69.2	9.8	8.7

All values are in $\mu g\ l^{-1}$.

These results suggest that the *E. dilatata* strain and enzyme systems studied could potentially be used as biomarkers of environmental pollution. However, further studies are required with this test organism and others populations of the same species. These include: (1) the use of molecular techniques to analyse species complexes (e.g. mitochondrial markers like Cox1); (2) biochemical analysis of other enzyme systems; (3) development of field or mesocosm studies; and (4) expanding the database on toxicants to evaluate their interaction with the toxicological sensitivity of *E. dilatata*. In the last case, it is worth mentioning the high concentrations of detergents, phenols, manganese, aluminium and iron that have been reported in sediments of San Pedro River (Mora 2007, Torres-Guzmán et al. 2010) where populations of *E. dilatata* could be present.

In conclusion, concentrations that show a significant inhibitory effect on the catalytic activity of esterases and PLA2s (EC_{50} values) are very close to lethal concentrations of evaluated toxicants. However, the results suggest that the enzyme systems in *E. dilatata* could be considered as good biomarkers of effect for metals and pesticides. Esterases are highly sensitive to cadmium and methyl parathion and PLA2s are very sensitive to mercury and lead. With respect to DDT, both biomarkers have the same sensitivity. Compared with other test organisms and endpoints, biochemical processes seem to be more sensitive toxicological endpoints than physiological processes such as food intake rate. Although the results correspond to a particular strain, high sensitivity shown for the test species could be used as bioindicator of environmental stress in sediments and littoral areas of aquatic ecosystems; it would allow estimation of the chronic effects caused by toxicants on populations or communities. Moreover, the results obtained suggest studies of possible differences in the susceptibility or tolerance of different strains of *E. dilatata* from Mexico.

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Declaration of interest

The authors report no declarations of interest.

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